

Expressions of RANKL/RANK and M-CSF/*c-fms* in root resorption lacunae in rat molar by heavy orthodontic force

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SUMMARY The differentiation and functions of osteoclasts are regulated by receptor activator of nuclear factor- κ B (RANK)/receptor activator of nuclear factor- κ B ligand (RANKL) system that stimulates osteoclasts formation. Macrophage colony-stimulating factor (M-CSF) is also essential for osteoclastogenesis. A recent immunocytochemical study reported that RANKL/RANK and M-CSF/*c-fms* were localized in the periodontal ligament of rat molars during experimental orthodontic tooth movement. The present study focused on the expressions of RANKL/RANK and M-CSF/*c-fms* in root resorption area during experimental tooth movement in rats.

Forty 6-week-old male Wistar rats were subjected to an orthodontic force of 10 or 50 g with a closed coil spring (wire size: 0.005 inch, diameter: 1/12 inch) ligated to the maxillary first molar cleat by a 0.008 inch stainless steel ligature wire to induce a mesial tipping movement of the upper first molars. Experimental tooth movement was undertaken for 10 days. Each sample was sliced into 6 μ m continuous sections in a horizontal direction and prepared for haematoxylin and eosin (H and E) and immunohistochemistry staining for tartrate-resistant acid phosphatase (TRAP), RANK, RANKL M-CSF, and *c-fms* in root resorption area. Statistical analysis was carried out using a Mann–Whitney *U*-test with a significance level of $P < 0.01$.

On days 7 and 10, immunoreactivity for RANKL/RANK and M-CSF/*c-fms* was detected in odontoclasts with an orthodontic force of 50 g, but not 10 g. Therefore, RANKL/RANK and M-CSF/*c-fms* systems may be involved in the process of root resorption by heavy orthodontic force.

Introduction

Two cytokines are essential for osteoclastogenesis, the receptor activator of nuclear factor- κ B ligand (RANKL; Lacey *et al.*, 1998; Yasuda *et al.*, 1998) and macrophage colony-stimulating factor (M-CSF), also called colony-stimulating factor-1, with the former being the key osteoclastogenic cytokine and the latter contributing to proliferation, survival, and differentiation of early precursors (Udagawa *et al.*, 1999). According to Soedarsono *et al.* (2006), recent advances in bone cell biology have allowed elucidation of the crucial roles of RANK/RANKL/osteoprotegerin (OPG) system in osteoclast differentiation and function. RANKL is a cytokine that belongs to the tumour necrosis factor (TNF) family and is essential for the induction of osteoclastogenesis. Osteoblasts and bone marrow stromal cells produce this cytokine, and its signals are transduced by the specific receptor RANK, which is localized on the cell surface of osteoclast progenitors. The RANKL/RANK system has been suggested to play an integral role in osteoclast activation during orthodontic tooth movement. Shiotani *et al.* (2001) observed RANKL in osteoblasts, osteocytes, fibroblasts, and osteoclasts during the application of orthodontic forces. The RANKL/RANK system may

regulate the natural process of root resorption in exfoliated primary teeth (Lossdörfer *et al.*, 2002).

M-CSF has also been identified as a molecule that mediates the survival and proliferation of precursors of the monocyte/macrophage lineage, as well as their differentiation into phagocytes (Hamilton and Anderson, 2004). The finding that cells of the myeloid lineage are osteoclast precursors suggests that M-CSF plays an important role in osteoclast biology. Cellular response to M-CSF is regulated by the M-CSF receptor, which is a single-chain molecule encoded by the *c-fms* proto-oncogene that consists of an extracellular ligand-binding domain, single membrane-spanning segment, and intercellular tyrosine kinase domain. There is some evidence of a role for M-CSF/*c-fms* system in osteoclast activation during orthodontic tooth movement. Kaku *et al.* (2008) observed the expression of M-CSF mRNA in osteoblasts and fibroblasts during experimental tooth movement. Kitauro *et al.* (2008) reported that the anti-*c-fms* antibody inhibited tooth movement by inhibiting osteoclastogenesis. M-CSF/*c-fms* system may involve the velocity of tooth movement (Yamaguchi *et al.*, 2007).

Orthodontically induced inflammatory root resorption (OIIRR) is an unavoidable pathological consequence of orthodontic tooth movement. It can be defined as an

iatrogenic disorder that occurs, unpredictably, after orthodontic treatment, whereby the resorbed apical root portion is replaced with normal bone. OIIR is a sterile inflammatory process that is extremely complex and involves various disparate components, including mechanical forces, tooth roots, bone, cells, surrounding matrix, and certain known biological messengers (Krishnan and Davidovitch, 2006; Meikle, 2006). A recent study demonstrated that mRNA of RANK was detected in tissues involved in root resorption following application of orthodontic forces (Low *et al.*, 2005). Furthermore, Yamaguchi *et al.* (2006) reported that compressed periodontal ligament (PDL) cells obtained from tissues with severe external apical root resorption produced a large amount of RANKL that up-regulated osteoclastogenesis *in vitro*. However, few studies have attempted to identify the concomitant expressions of RANKL/RANK and M-CSF/*c-fms* during root resorption triggered by orthodontic force.

In the present study, the expressions of RANKL/RANK and M-CSF/*c-fms* in rat root resorption during experimental tooth movement were investigated using immunohistochemical analysis.

Materials and methods

Animals

The animal experimental protocol in this study was approved by the Ethics Committee for Animal Experiments at Nihon University School of Dentistry at Matsudo (approval No. ECA-05-0025).

A total of 40 male, 6-week-old, Wistar strain rats (Sankyo Labo Service Co., Tokyo, Japan) weighting 180 ± 10 g were used for the experiment. The rats were divided into eight groups (five rats each), according to the magnitude and duration of the applied force. Four time groups were set at 0, 3, 7, and 10 days and two magnitude groups at 10 and 50 g. The contralateral molars served as the controls (0 g). The rats were kept in the animal centre of Nihon University School of Dentistry at Matsudo in separate cages, in a 12 hour light/dark environment at a constant temperature of 23°C, and provided with food and water *ad libitum*. The health status of each rat was evaluated by daily body weight monitoring for 1 week before the start of the experiments.

Application of orthodontic devices and tissue harvesting

The animals were anaesthetized with ketamine (90 mg/kg body weight) and rompam (10 mg/kg body weight) for insertion of orthodontic devices. Experimental tooth movement was performed using the method of Fujita *et al.* (2008), with a closed coil spring (wire size: 0.005 inch, diameter: 1/12 inch; Accurate Sales Co., Chiba, Japan) ligated to the maxillary first molar cleat by a 0.008 inch stainless steel ligature wire (Tomy International Inc., Tokyo,

Japan). The other side of the coil spring was also ligated, with the holes in the maxillary incisors drilled laterally just above the gingival papilla with a no. 1/4 round bur, using the same ligature wire. The upper first molar was moved mesially by the closed coil spring with a force of 10 or 50 g (Figure 1). The period of the experiment was 10 days.

Measurement of tooth movement

Measurement of tooth movement was performed according to the method of Fujita *et al.* (2008). To determine the amount of tooth movement, plaster models of the maxillae were made using a silicone impression material (Dent Silicone-V; Shofu Inc., Kyoto, Japan) before (day 0) and after tooth movement (days 3, 7, or 10). The models were scanned using a contact-type three-dimensional (3D) measurement apparatus (3D-picza; Roland Co., Hamamatsu, Japan) by setting the plane to pass through three points, i.e. the bilateral interpapillary crests between the first and second molars and the interpapillary crest between the second and third molars.

Using 3D morphological analysis software (3D-Rugle; Medic Engineering Inc., Kyoto, Japan), the distance between the central fossa of the first molar and the mesial surface of the second molar was measured to determine tooth movement.

Tissue preparation

The experimental periods were set at 0, 3, 7, and 10 days after tooth movement. The animals were deeply anaesthetized by pentobarbital sodium and perfused with 4 per cent paraformaldehyde in 0.1 M phosphate buffer transcardially, after which the maxilla was immediately dissected and immersed in the same fixative overnight at 4°C. The specimens were decalcified in 10 per cent disodium ethylenediaminetetraacetic acid (pH 7.4) solution for 4

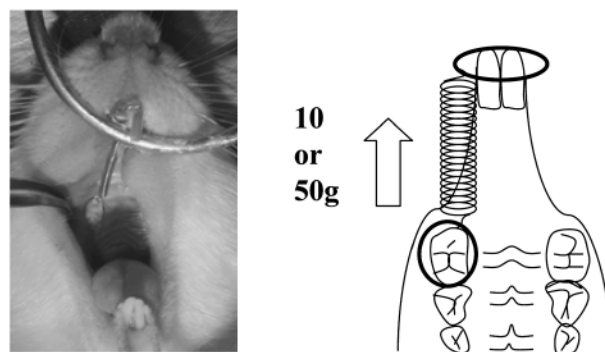


Figure 1 Experimental tooth movement was performed with a closed coil spring (wire size: 0.005 inch, diameter: 1/12 inch) ligated to the maxillary first molar cleat by a 0.008 inch stainless steel ligature wire. The other side of the coil spring was also ligated, with the holes in the maxillary incisors drilled laterally just above the gingival papilla with a no. 1/4 round bur, using the same ligature wire. The upper first molar was moved mesially by the closed coil spring at 10 or 50 g. The period of the experiment was 10 days.

weeks and then the decalcified specimens were dehydrated through an ethanol series and embedded in paraffin. Each sample was sliced into 6 µm continuous sections in the horizontal direction and prepared for haematoxylin and eosin (H&E) and immunohistochemistry staining for tartrate-resistant acid phosphatase (TRAP), RANK, RANKL, M-CSF, and *c-fms*. The periodontal tissues in the mesial part of the mesial root of an upper first (M1) were observed.

Immunohistochemistry

Immunohistochemical staining was performed as follows: the sections were deparaffinized and endogenous peroxidase activity was quenched by incubation in 3 per cent H₂O₂ in methanol for 30 minutes at room temperature. After washing in Tris-buffered saline (TBS), the sections were incubated with polyclonal anti-rabbit TRAP (Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA; working dilution, 1:400), polyclonal anti-rabbit RANK (Santa Cruz biotechnology; working dilution, 1:100), polyclonal anti-goat RANKL (Santa Cruz Biotechnology; working dilution, 1:100), polyclonal anti-rabbit M-CSF (Santa Cruz Biotechnology; working dilution: 1:300), and polyclonal anti-rabbit *c-fms* (Santa Cruz Biotechnology; working dilution: 1:300) overnight at 4°C. TRAP, RANK, M-CSF, and *c-fms* were stained using Histofine Simple Stain MAX-Po kits (Nichirei, Tokyo, Japan), and RANKL was stained using a Histofine SAB-PO (G) kit (Nichirei) according to the manufacturer's protocol.

The sections were rinsed with TBS and final colour reactions were performed using the substrate reagent 3, 3'-diaminobenzidine tetra-hydrochloride and aminoethylcarbazole and then counter-stained with haematoxylin. As immunohistochemical controls, some sections were incubated in the same way and then with either non-immune rabbit IgG or 0.01 M (phosphate-buffered saline) alone, instead of the primary antibody.

Quantitative assessment

Quantitative measurements were conducted at the root-PDL-alveolar bone complex close to the apical root, where orthodontic root resorption usually occurs (Chutimanutskul *et al.*, 2006). The slides were evaluated at a magnification of ×400 with a fixed measurement frame (450 × 450 µm). TRAP-positive cells were counted as multinucleate odontoclasts, which were observed on the surface of cementum. The data were then processed with a Mann-Whitney *U*-test. A value of *P* < 0.01 was considered to indicate a significant difference.

Results

Tooth movement during the experimental period

The body weight of the rats in both force groups decreased transiently on day 1 and then recovered. No significant

differences between the two groups were observed (data not shown). The amount of tooth movement also was not significantly different between the two groups on day 10 (data not shown).

Histological changes in periodontal tissues during tooth movement (H&E staining)

In the control group (0 g) on days 3 and 7 after tooth movement, PDL specimens were composed of relatively dense connective tissue fibres and fibroblasts that regularly ran in a horizontal direction from the root cementum towards the alveolar bone. Blood capillaries were mainly recognized near the alveolar bone in the PDL. The alveolar bone and root surface were relatively smooth, with a few mononuclear and multinucleate osteoclasts, and resorption lacunae were observed on the alveolar bone surface (Figure 2a and 2b).

In the 10 g group, the arrangement of the fibres and fibroblasts became coarse and irregular, and blood capillaries were compressed on day 3. Resorption lacunae with a few multinucleate osteoclasts were observed on the surface of the alveolar bone surface (Figure 2c). On day 7, on the surface of the alveolar bone, bone resorption lacunae with multinucleate osteoclasts were observed. Osteoclasts on the alveolar bone were increased in comparison with those on day 3 (Figure 2d).

The PDL in the 50 g group was composed of a coarse arrangement of fibres and expanded blood capillaries. Many resorption lacunae with multinucleate osteoclasts were observed on the alveolar bone on day 3 (Figure 2e). On day 7, many root resorption lacunae with multinucleated odontoclasts were recognized on the surface of the root (Figure 2f).

TRAP immunohistochemical findings

In the control group (0 g) on days 3 and 7, a few resorption lacunae with TRAP-positive multinucleated osteoclasts were observed on the surfaces of the alveolar bone and root (Figure 3A-a and b).

In the 10 g group, some resorption lacunae with TRAP-positive multinucleated osteoclasts appeared on the surface of the alveolar bone on day 3 (Figure 3A-c). On day 7, on the alveolar bone surface, bone resorption lacunae with multinucleated TRAP-positive osteoclasts were recognized. The multinucleate TRAP-positive osteoclasts on the alveolar bone were increased in comparison with those on day 3 (Figure 3A-d).

In the 50 g group, on the surface of the alveolar bone, many bone resorption lacunae with multinucleate TRAP-positive osteoclasts were observed on day 3 (Figure 3A-e) and TRAP-positive odontoclasts on day 7 (Figure 3A-f).

Quantitative evaluation showed the number of TRAP-positive odontoclasts to be significantly increased in the 50 g group on day 7 (6.0-fold) as compared with the 10 g group (**P* < 0.01, Figure 3B).

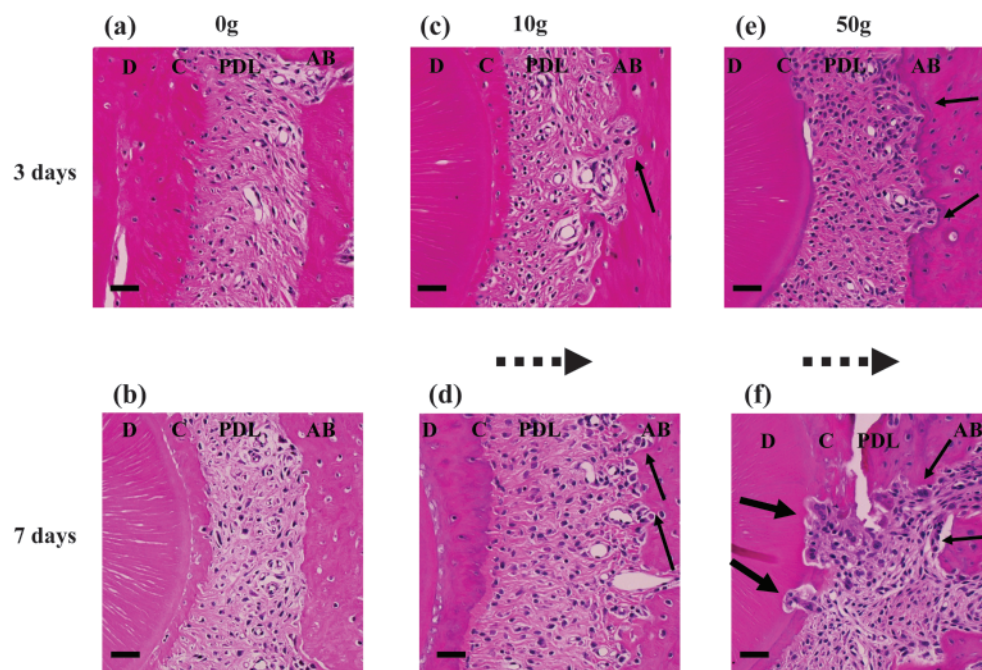


Figure 2 Light microscopic images of the effect of different orthodontic forces on the multinucleate osteoclasts (haematoxylin and eosin; original magnification $\times 400$). Osteoclasts (thin arrows) appeared on the alveolar bone surface in both groups on days 3 (c and e) and 7 (d and f). Odontoclasts (thick arrows) on the cementum in the 50 g group was more than that of the 10 g on day 7 (f). AB: alveolar bone, PDL: periodontal ligament, C: cementum, and D: dentine; bar = 50 μ m. The direction of applied force is indicated by the dotted arrow.

Protein expressions of RANKL/RANK and M-CSF/*c-fms*

Immunoreactivity for RANKL/RANK and M-CSF/*c-fms* was performed on days 7 and 10 after tooth movement. In the control (0 g) group, the immunoreactivity of RANKL and M-CSF were weakly localized in cytoplasm of some fibroblasts and pericytes near the alveolar bone surface (Figures 4A-a, 4B-a, 5A-a, and 5B-a). RANK- and *c-fms*-positive osteoclasts were not observed (Figures 4A-b, 4B-b, 5A-b, and 5B-b). In the 10 g group, a few RANKL- and M-CSF-positive fibroblasts in the PDL and osteoblasts at the root surface were increased (Figures 4A-c, 4B-c, 5A-c, and 5B-c). RANK- and *c-fms*-positive odontoclasts were rarely observed (Figures 4A-d, 4B-d, 5A-d, and 5B-d). In the 50 g group, many RANKL- and M-CSF-positive odontoclasts and fibroblasts were observed (Figures 4A-e, 4B-e, 5A-e, and 5B-e). Many odontoclasts showed strong immunoreactivity of RANK and *c-fms* (Figures 4A-f, 4B-f, 5A-f, and 5B-f).

Discussion

Considering the method used to induce root resorption, many resorption lacunae with multinucleated osteoclasts and odontoclasts appeared on the alveolar bone and root on days 3 and 7 after tooth movement (Figures 2 and 3). Many investigators have reported that root resorption is aggravated by increasing force magnitudes (Reitan, 1985; Chan and

Darendeliler, 2005). Gameiro *et al.* (2008) demonstrated osteoclastic resorption of roots on the mesial surfaces of teeth subjected to heavy orthodontic force (50 g). Gonzales *et al.* (2008) showed that 10 g of light force application produced significantly greater tooth movement with significantly less root resorption over a period of 28 days in relation to heavier force application in rats. The optimum force for the movement of the rat upper molar may be less than the 10 g previously suggested (Kohno *et al.*, 2002).

On days 7 and 10, immunoreactivity for RANKL/RANK was detected in odontoclasts in the 50 g group (Figure 4A and 4B). Oshiro *et al.* (2001) suggested that osteoblast/stromal cells and PDL fibroblasts are involved in supporting osteoclast differentiation during tooth movement. Further, Low *et al.* (2005) reported that mRNA of RANKL was detected in tissues including root resorption subjected to orthodontic forces. Taken together, those findings and the present results suggest that osteoclast and odontoclasts differentiation in root resorption areas are critically regulated by RANKL, which is produced as a local factor by PDL cells in response to heavy orthodontic force. RANKL has been identified to be an osteoclast differentiation factor that binds to RANK on the cell surface of osteoclastic cells (Myers *et al.*, 1999). On the other hand, OPG is a novel secreted member of the TNF receptor superfamily that works as a decoy receptor of the RANKL–RANK signalling

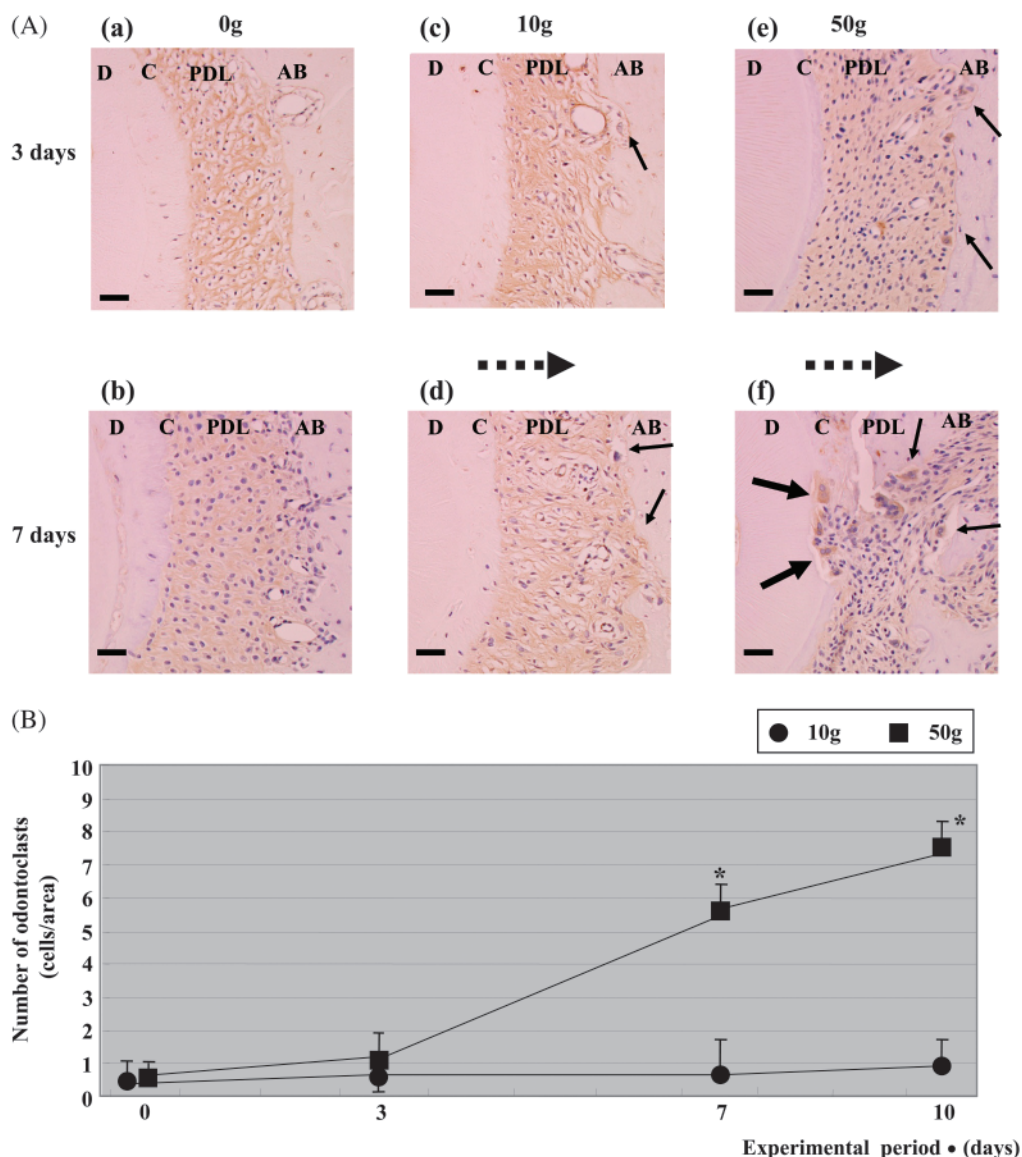


Figure 3 (A) Effect of different orthodontic forces on tartrate-resistant acid phosphatase (TRAP)-positive odontoclasts by immunohistochemistry (original magnification $\times 400$). The osteoclasts (thin arrows) appeared on the alveolar bone surface in both groups on days 3 (c and e) and 7 (d and f). Immunoreactivity of TRAP positive was observed in the odontoclasts (thick arrows) on the cementum in the 50 g group (f), but not in the 10 g on day 7. AB: alveolar bone, PDL: periodontal ligament, C: cementum, and D: dentine; bar = 50 μ m. The direction of applied force was indicated by the dotted arrow. (B) Diagram depicting quantitative assessment of cellular changes. The number of TRAP-positive odontoclasts in the 50 g group was more than in the 10 g on days 7 and 10 (* $P < 0.01$).

system, thereby inhibiting osteoclastogenesis (Burgess *et al.*, 1999). Thus, OPG, RANKL, and RANK form a critical system that controls bone resorption by regulating the number and activity of osteoclasts. In relation to the OPG/RANKL/RANK system and orthodontic tooth movement, since RANKL expression was observed in compressed PDL cells, it is possible that osteoclastogenesis is promoted on the compression side during orthodontic tooth movement (Garlet *et al.*, 2007). Oshiro *et al.* (2001) reported that in OPG-deficient mice, there was a greater induction of TRAP-positive multinucleated cells in the PDL

on the compressed side and in the adjacent alveolar bone after experimental application of orthodontic force than in their wild-type OPG (+/+) littermates. Kanzaki *et al.* (2004) stated that OPG gene transfer to periodontal tissue inhibited RANKL-mediated osteoclastogenesis and inhibited experimental tooth movement. Nishijima *et al.* (2006) found an increased concentration of RANKL in gingival crevicular fluid (GCF) during orthodontic tooth movement, and the ratio of concentration of RANKL to that of OPG in the GCF was significantly higher than at the control sites. These studies suggest that during orthodontic tooth movement, the

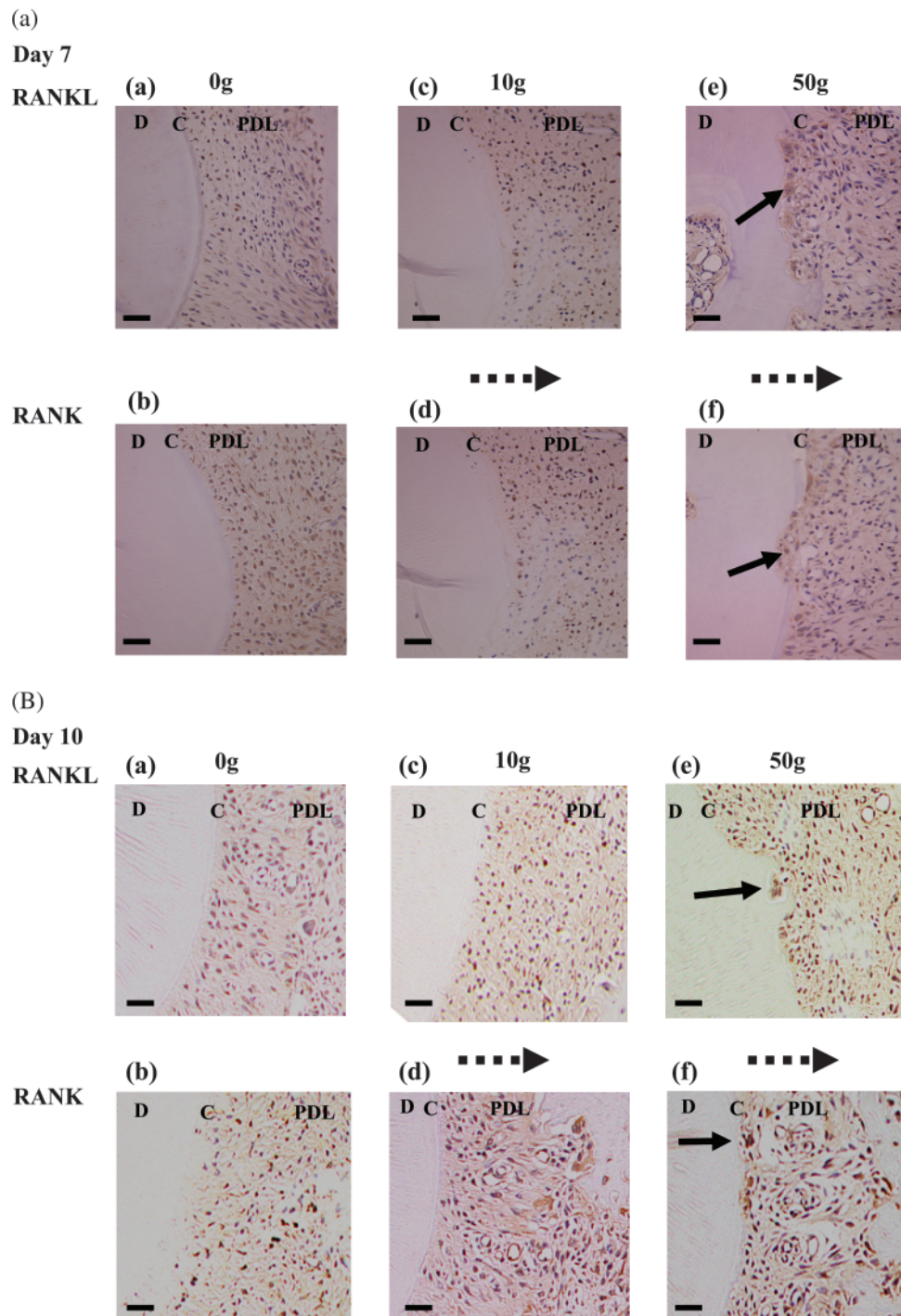


Figure 4 Effect of different orthodontic forces on RANKL- and RANK-positive odontoclasts by immunohistochemistry on days 7 (Figure 4A) and 10 (Figure 4B; original magnification $\times 400$). Immunoreactivity of RANKL and RANK was observed in the odontoclasts (arrow) on the cementum in the 50 g group (4AB-e, f), but not in the 10 g (4AB-c, d) on days 7 and 10. PDL: periodontal ligament, C: cementum, D: dentine; bar = 50 μ m. The direction of applied force is indicated by the dotted arrow.

OPG/RANKL/RANK system in the periodontal tissues is an important determinant regulating balanced alveolar bone resorption. Taken together, those findings and the present results suggest that the OPG/RANKL/RANK system may play an important role in orthodontically induced root

resorption. Further studies are necessary to confirm the relationship between OPG and RANKL/RANK in root resorption.

On the other hand, M-CSF stimulates not only the proliferation of osteoclast progenitors but also their

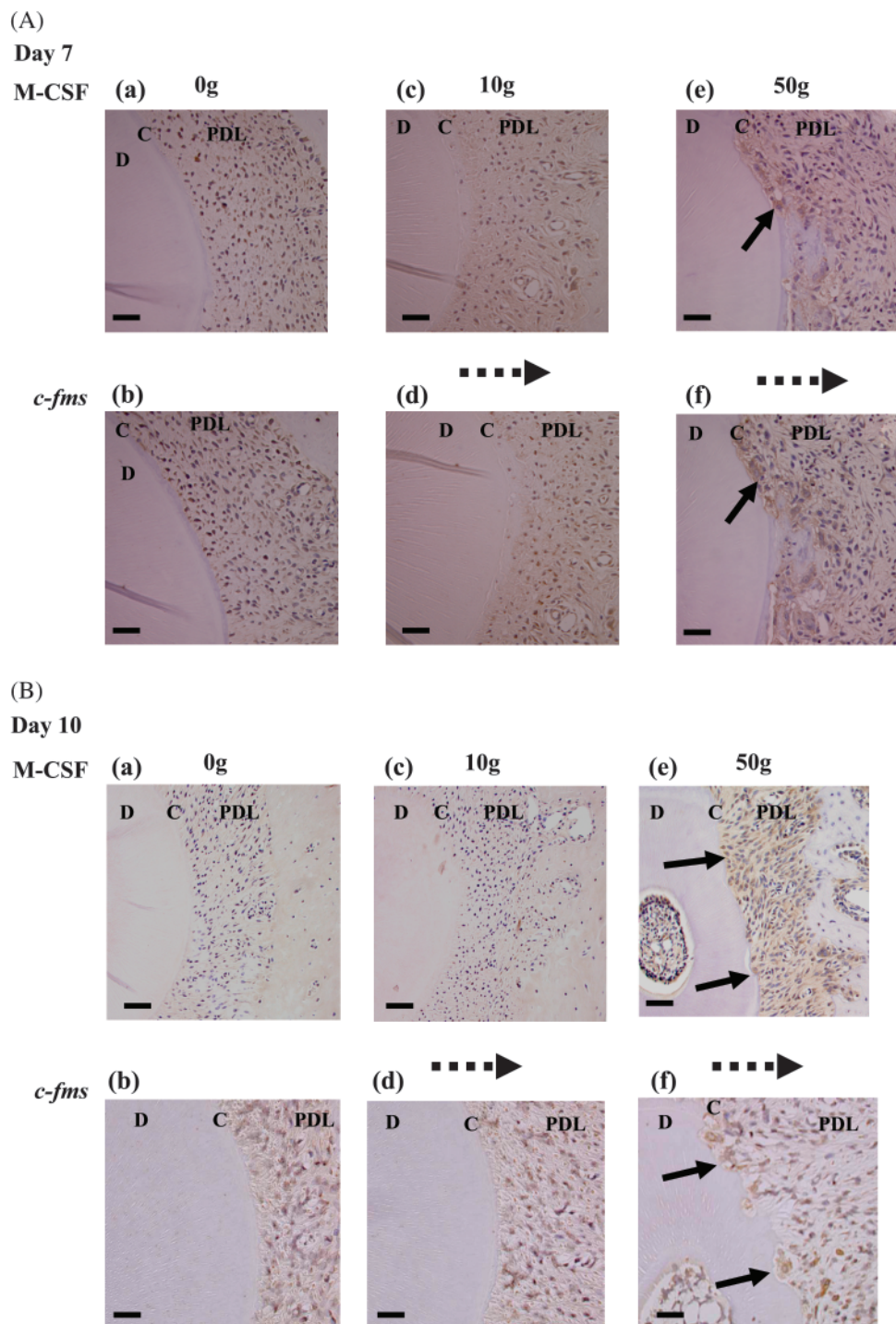


Figure 5 Effect of different orthodontic forces on macrophage colony stimulating factor (M-CSF)- and *c-fms*-positive odontoclasts by immunohistochemistry on days 7 (Figure 4A) and 10 (Figure 4B; original magnification $\times 400$). Immunoreactivity of M-CSF and *c-fms* were observed in the odontoclasts (arrows) on the cementum in the 50 g group (4AB-e, f), but not in the 10 g (4AB-c, d) on days 7 and 10. PDL: periodontal ligament, C: cementum, D: dentine; bar = 50 μ m. The direction of applied force is indicated by the dotted arrow.

differentiation into mature osteoclasts *in vivo* (Felix *et al.*, 1994) and *in vitro* (Tanaka *et al.*, 1993). Yang *et al.* (1996) reported that the presence of the M-CSF receptor on preformed osteoclasts is carried over from the developing

cells and plays a functional role in the modulation of bone resorption by mature cells. *c-fms*, the cellular homologue of the feline transforming virus v-FMS, is the sole receptor for M-CSF (Stanley *et al.*, 1997). The functional linkage

between M-CSF and its receptor has been established by the finding that mice lacking *csflr*, the gene coding for *c-fms*, exhibit the same major phenotype as *op/op* mice, namely a marked decrease in tissue macrophages and severe osteopetrosis due to a lack of osteoclasts (Dai *et al.*, 2002).

Previous studies have demonstrated that a severe deficiency of osteoclasts in *op/op* mice can be reversed by injections of recombinant human M-CSF (Kodama *et al.*, 1991a; Wiktor-Jedrzejczak *et al.*, 1991), and the direct action of M-CSF on osteoclast lineage cells was exhibited by expression of the receptors for M-CSF and *c-fms* in osteoclasts both *in vitro* (Kodama *et al.*, 1991b) and *in vivo* (Hofstetter *et al.*, 1995). Those findings indicate that M-CSF plays an essential role in the differentiation of osteoclasts. In addition, it has become clear that M-CSF supports osteoclast differentiation in co-operation with RANKL (Lacey *et al.*, 1998; Yasuda *et al.*, 1998; Udagawa *et al.*, 1999). Kimoto *et al.* (1999) suggested that M-CSF may affect bone remodelling and root resorption in PDL cells derived from human primary teeth in response to mechanical stress *in vitro*. On days 7 and 10, immunoreactivity for M-CSF/*c-fms* was also detected in odontoclasts in the 50 g group (Figure 5A and 5B). Therefore the M-CSF and *c-fms* system may also play an important role in orthodontic root resorption.

Conclusions

On days 7 and 10, immunoreactivity for RANKL/RANK and M-CSF/*c-fms* was detected in rat odontoclasts and PDL fibroblasts with an orthodontic force of 50 g. Therefore, RANKL/RANK and M-CSF/*c-fms* systems may be involved in the process of root resorption by excessive orthodontic force.

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